

A SURVEY OF THE ANTIMYCOTIC AND ANTIBACTERIAL ACTIVITY OF SOIL MICROFUNGI FROM TRANSVAAL.

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ABSTRACT

One hundred and forty-two species of microfungi from a Transvaal soil were examined for the production of antifungal and antibacterial substances. Two methods of assay were used viz. a streak method on agar plates and a liquid culture method.

Antibiotic substances were produced by 74 species (52%). The most significant genera were *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium* and *Trichoderma*.

Evidence was found to support the view that the capacity to produce antifungal and antibacterial substances is of ecological significance and that this ability is associated with the dominant fungi in the soil.

UITTREKSEL

'N ONDERSOEK VAN DIE ANTIFUNGALE- EN ANTIBAKTERIESE-AKTIWITEITE VAN GRONDMIKROFUNGI IN TRANSVAAL.

Een honderd twee-en-veertig mikrofungi van 'n Transvaalse grond is getoets vir die produksie van antifungale- en antibakteriese-stowwe. Twee toetsmetodes is gebruik naamlik uitstryking op agarplate en 'n vloeibare kultuurmedium-tegniek.

Antibiotiese stowwe is deur 74 spesies (52%) gevorm. Die mees opvallende genera was *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium* en *Trichoderma*.

Bewys is gevind wat die opvatting ondersteun dat die vermoë om antifungale- en antibakteriese-stowwe voort te bring van ekologiese belang is en dat hierdie vermoë in verband staan met die voorkoms van die dominante fungi in die grond.

INTRODUCTION

Many soil inhabiting fungi produce inhibitory substances in laboratory media which antagonize a variety of bacteria, fungi and actinomycetes. Despite the high proportion of soil fungi producing antibiotics in culture, the role of these organisms in the population equilibrium and their significance in determining the composition of the soil microflora is not yet clear. There has been considerable speculation about the ecological significance of antibiotic production by soil-micro-organisms (Brian, 1957; Alexander, 1967). No relationship has yet been given between the predominant species in the soil and their sensitivity or resistance to antibiotics. The view that antibiotics does not play a major role in regulating the microbiological equilibrium has been based upon the following facts: that antibiotics never accumulate in the soil, except within the organic substrate; that they have a tendency to be inactivated by absorption

on colloids and that they are susceptible to biological decomposition (Wright, 1956; Krywolap *et al.*, 1964). None of these factors, however, eliminate the possible micro-ecological significance of antibiosis. Alexander (1967) suggests the following as evidence of the importance of antibiotic-producers in soil:

- (a) The abundance of soil species which inhibit the growth of test species;
- (b) the greater persistence in soil of indigenous than of alien fungi in the presence of antibiotic-producers;
- (c) those *Streptomyces* having the greatest antibiotic potency against certain test fungi in laboratory media generally having the most marked effect on the fungi when tested in sterile soil; and
- (d) the formation of antibiotics in normal soil supplemented with one or more per cent organic matter.

Jackson (1965), in his extensive review of the problem of antibiosis and fungistasis of soil micro-organisms, concluded that: "A consideration of all the evidence we now have must convince us that antibiosis, in one form or another, plays a key role in the ecology of soil micro-organisms, being perhaps second in importance only to competition for nutrients."

An ecological investigation of the mycoflora of an alkaline soil of the open-savannah of the Transvaal was conducted during 1971 and 1972. This paper reports on antibiotic production by the isolated soil fungi of the investigated site.

MATERIAL AND METHODS

Soil samples were obtained from an area of undisturbed, natural vegetation within the municipal area of Pretoria. The soil is an alkaline loam soil on which a Sourish Mixed Open-Savannah type vegetation (Acocks, 1953) occurs. The details of the soil analysis, vegetation, collection of soil samples as well as the methods employed for the isolation of the fungi are given elsewhere (Eicker, 1974).

The antifungal and antibacterial properties of the isolated fungi were examined using two methods. The first, a streak method on agar plate cultures (Pridham, 1956), screened all the isolates initially for antagonism. In the second method, the fungi were grown on liquid media and the culture filtrates were assayed periodically for antibiotic activity. The details of these methods are given below.

Streak test for antagonism in agar culture

Petri dishes were prepared containing 15 ml of 2.5% Bacto Antibiotic Medium 2 (Difco Penassay Agar). The medium contained the following:

Bacto-Beef extract 1,5 g; Bacto-Yeast extract 3 g; Bacto-Peptone 6 g; Agar-15 g; distilled water 1 000 ml. The plates were inoculated with a transverse streak of spores of each soil fungus, or with a mycelial transplant in cases where spores were not available. These seeded plates were incubated for 4 days at 25 °C. By means of a platinum loop, 24 hr. old broth cultures of three test bacteria and one test fungus, were then streaked at right angles to the advancing front of the fungus colony. The soil fungi were tested for antagonism to the fungus *Candida albicans* (Robin) Berkhout and the pathogenic bacteria *Staphylococcus aureus* Rosenbach, *Salmonella typhosa* Zopf and *Escherichia coli* Migula. The plates were incubated for a further 24 hr. at 35 °C, after which the degree of antagonism could be assessed in terms of the presence or absence of a clear zone adjacent to the fungus colony.

Antagonism is recorded in Table 1 as follows (Brian & Hemming, 1947):

- = no indication of antagonism,
- +
- ++ = zone of inhibition greater than 0,5 cm,
- (+) = an apparent decrease in growth of the test organism without production of a clear zone.

Assay of culture filtrates for antagonism

Fungi which proved to be strongly antagonistic in the streak method were tested further. They were grown in 250 ml Erlenmeyer flasks each containing 50 ml of liquid medium comprising a 2% Malt extract (Difco) solution. The flasks were agitated on an orbital shaker at 25 °C for 10 days. The liquid medium was filtered off and assayed for antagonism by the paper disc method (Loo, 1945). Twenty ml of sterile 2,5% Penassay agar per petri dish was allowed to gel before 10 ml of cooled agar seeded with *Bacillus subtilis* Cohn was then poured evenly over the surface. The *B. subtilis* cultures for seeding the agar were grown for 24 hr. at 25 °C on nutrient agar slants and then suspended in 0,83% NaCl solution. The concentration of the suspension was then adjusted with an Unigalvo Type 20 Nephelometer to obtain a uniform density of the bacteria. Three filter paper discs (Whatman Antibiotic Assay discs, 6 mm), previously impregnated with the filtrate of the soil fungus, were placed flat side down on the seeded agar. The cultures were incubated at 25 °C for 22 hr. after which the halos were measured to the nearest 0,1 mm. Only the zone of complete inhibition was considered. The mean values of six inhibition zones of each fungus tested are given in Table 1.

TABLE 1.
 Antibacterial and Antifungal activity of Transvaal Soil Fungi

Accession no.	Organism	Antagonistic effects in agar cultures. (See text for explanation of symbols.)				Antibiotic effect of culture filtrates
		Antibacterial effects			Antifungal effect	<i>Bacillus subtilis</i> . (Expressed in mm of zone of inhibition)
	Phycomycetes Zygomycetes	<i>Sta- phyllo- coccus</i>	<i>Esche- richia</i>	<i>Salmo- nella</i>	<i>Candida</i>	
440	<i>Absidia cylindrospora</i> Hagem .	+	—	++	—	23,0
478	<i>Absidia spinosa</i> Lendner . . .	—	—	(+)	—	*
625	<i>Mucor fragilis</i> Bainier . . .	—	—	+	+	
602	<i>Mucor racemosus</i> Fres. . . .	—	—	—	(+)	
492	<i>Mucor spinosus</i> van Tieghem .	+	—	+	+	15,4
331	<i>Chaetomium globosum</i> var. <i>flavo- viride</i> Novak	+	—	—	—	
700	<i>Chaetomium lusitanicum</i> Gomes	++	—	—	—	0
433	<i>Gymnoascus umbrinus</i> (Boudier) Orr, Kuehn & Pluckitt . . .	++	—	—	(+)	19,6
716	<i>Nectria invecta</i> Pethybr. . . .	++	—	—	—	0
	Fungi Imperfecti (Deuteromycetes) Sphaeropsidales					
723	<i>Coniella diplodiella</i> (Speg.) Petrak & Sydow	+	—	—	—	0
						* Culture filtrates not assayed
502	<i>Phoma</i> sp.	—	—	—	(+)	
1059	<i>Phoma</i> sp.	+	—	—	++	13,3
790	Unidentified Sphaeropsidales .	—	—	—	+	
1040	Unidentified Sphaeropsidales .	—	—	++	+	0
	Melanconiales					
313	<i>Pestalotia</i> sp.	—	—	—	+	
797	<i>Pestalotia</i> sp.	—	—	—	++	21,5
	Moniliaceae					
616	<i>Aspergillus aculeatus</i> Iizuka . .	(+)	—	—	(+)	0
412	<i>Aspergillus carneus</i> (Van Tieg- hem) Blockwitz	++	—	—	(+)	0
445	<i>Aspergillus</i> (Bain & Sart.) Thom & Church	++	—	—	+	30,2

Accession no.	Organism	Antagonistic effects in agar cultures. (See text for explanation of symbols.)				Antibiotic effect of culture filtrates
		Antibacterial effects			Anti-fungal effect	<i>Bacillus subtilis</i> . (Expressed in mm of zone of inhibition)
		<i>Staphylococcus</i>	<i>Escherichia</i>	<i>Salmonella</i>	<i>Candida</i>	
	Phycomycetes Zygomycetes					
	Moniliaceae (Cont.)					
320	<i>Aspergillus flavus</i> (Link) Fr. . .	++	—	++	+	28,6
338	<i>Aspergillus fumigatus</i> Fres. . .	++	—	—	—	0
509	<i>Aspergillus nidulans</i> (Eidam) Wint. . .	—	—	—	(+)	27,6
528	<i>Aspergillus ochraceus</i> Wilhelm . .	++	+	+	+	12,2
324	<i>Aspergillus rugulosus</i> Tom & Raper . .	+	—	—	++	11,0
516	<i>Aspergillus ustus</i> (Bain.) Thom & Church . .	—	—	(+)	(+)	0
342	<i>Aspergillus versicolor</i> (Vuill.) Tiraboschi	+	—	—	+	0
410	<i>Aspergillus</i> sp.	—	—	—	—	0
708	<i>Aspergillus</i> sp.	+	—	—	—	0
468	<i>Gliocladium</i> cf. <i>roseum</i> Bain. agg. . .	—	—	(+)	+	0
450	<i>Paecilomyces</i> sp.	(+)	+	—	—	0
820	<i>Paecilomyces</i> sp.	+	—	—	—	0
514	<i>Penicillium brevicompactum</i> Dierckx	—	—	—	(+)	17,8
351	<i>Penicillium chrysogenum</i> Thom . .	++	+	+	(+)	0
762	<i>Penicillium crustosum</i> Thom . .	+	—	+	—	33,0
760	<i>Penicillium cyclopium</i> Westling . .	+	++	++	(+)	0
609	<i>Penicillium herquei</i> Bain. & Sart. . .	+	—	—	—	0
435	<i>Penicillium lilacinum</i> Thom . .	+	—	—	—	0
493	<i>Penicillium multicolor</i> G.M.—P. . .	—	—	—	++	0
411	<i>Penicillium thomii</i> Maire	+	—	+	—	0
480	<i>Penicillium waksmanii</i> Zal. . . .	+	—	—	—	0
458	<i>Penicillium</i> sp.	++	—	—	—	0
465	<i>Penicillium</i> sp.	—	—	—	(+)	0
695	<i>Penicillium</i> sp.	+	—	+	—	0
770	<i>Penicillium</i> sp. (Ascosporous) (IMI 161 963)	++	—	—	—	29,2
793	<i>Penicillium</i> sp.	—	++	++	—	15,3
1039	<i>Penicillium</i> sp.	—	—	++	++	0
1041	<i>Penicillium</i> sp.	—	—	++	—	7,3
1085	<i>Penicillium</i> sp.	(+)	—	++	(+)	0
1098	<i>Penicillium</i> sp.	++	—	—	—	0
1128	<i>Penicillium</i> sp.	+	—	—	+	15,7
1168	<i>Penicillium</i> sp.	—	—	++	+	18,9
438	<i>Spicaria violacea</i> Abbott. . . .	++	—	—	+	26,33
717	<i>Trichoderma koningii</i> Oud. . . .	++	++	++	++	30,00
329	<i>Trichoderma pseudokoningii</i> Rifai agg.	++	++	++	++	30,00

Accession no.	Organism	Antagonistic effects in agar cultures. (See text for explanation of symbols.)				Antibiotic effect of culture filtrates
		Antibacterial effects			Antifungal effect	<i>Bacillus subtilis</i> . (Expressed in mm of zone of inhibition)
	Phycomycetes Zygomycetes	<i>Staphylococcus</i>	<i>Escherichia</i>	<i>Salmonella</i>	<i>Candida</i>	
	Moniliaceae (Cont.)					
653	<i>Trichoderma</i> cf. <i>pseudokoningii</i> Rifai agg.	—	—	—	+	
1116	<i>Trichoderma viride</i> (Pers.) Gray	++	—	++	++	19,4
1012	<i>Trichoderma</i> sp.	++	++	++	++	8,33
1016	<i>Trichoderma</i> sp.	++	++	++	++	19,33
1028	<i>Trichoderma</i> sp.	+	—	++	++	19,63
1066	<i>Trichoderma</i> sp.	++	+	++	—	15,73
1077	<i>Trichoderma</i> sp.	—	+	+	++	0
1120	<i>Trichoderma</i> sp.	++	—	+	—	0
1130	<i>Trichoderma</i> sp.	++	—	—	—	
696	<i>Verticillium</i> sp.	+	—	—	—	
1107	<i>Verticillium</i> sp.	++	—	—	—	
1119	<i>Verticillium</i> sp.	+	+	—	—	
	Dematiaceae					
726	<i>Alternaria tenuissima</i> (Kunze ex Pers.) Wiltshire	+	—	+	—	
1027	<i>Alternaria</i> sp.	+	—	—	++	0
792	<i>Cladosporium oxysporum</i> Berk. & Curt.	—	—	—	+	
1065	<i>Cladosporium</i> sp.	+	—	—	+	
1114	<i>Cladosporium</i> sp.	+	—	—	—	
1016	<i>Curvularia</i> sp.	—	—	—	++	6,9
	<i>Strachybotrys atra</i> Corda	+	—	+	(+)	
475	<i>Fusarium equiseti</i> (Corda) Sacc.	—	—	—	+	
418	<i>Fusarium oxysporum</i> Schlecht	—	(+)	—	++	0
720	<i>Fusarium oxysporum</i> Schlecht	—	—	—	(+)	
745	<i>Fusarium roseum</i>	—	—	—	++	
511	<i>Fusarium sambucinum</i> Fuckel	—	—	—	(+)	
456	<i>Fusarium solani</i> (Mart.) Sacc.	++	—	—	(+)	19,0
806	<i>Fusarium solani</i> (Mart.) Sacc.	—	—	—	+	
759	<i>Fusarium</i> sp.	++	—	—	(+)	0
1075	<i>Fusarium</i> sp.	—	—	—	+	
	Stilbaceae					
319	<i>Graphium putredinis</i> (Corda) Hughes	—	—	—	+	
727	Sterile culture	++	—	—	+	9,0

RESULTS AND DISCUSSION

The ability to produce antifungal substances in the various taxonomic groups of fungi is given in Table 2. Antimycosis was displayed by 2 Zygomycetes, 6 Aspergilli, 2 Cladosporia, 5 Fusaria, 5 Penicillia, 8 Trichodermae and 12 other Fungi Imperfecti. Not one of the Ascomycetes assayed showed any activity against *C. albicans*. The antifungal activity of the *Fusarium* species are of interest. Twelve species were assayed of which 5 showed strong antifungal activity and a further 4 caused less inhibition of the fungal growth. The *Trichoderma* species were particularly active in their antifungal characteristics. Weindling (1941) attributed the antagonistic powers of *Trichoderma* to the secretion of a lethal toxin named gliotoxin. Many strains of *Trichoderma* apparently also produce a powerful antifungal substance viridin (Brian, 1946). Webster and Lomas (1964), however, questioned the production of gliotoxin and viridin by members of this genus. In 1971 Dennis and Webster showed conclusively that these two antibiotics were not produced by *Trichoderma* species, but that chloroform soluble antibiotics, such as trichodermin and certain peptide antibiotics, are formed instead.

Blunt and Baker (1968) found that 35% of the 600 species of fungi isolated from 100 forest and cultivated soils in Hawaii inhibited fungal growth. In this study 142 species of soil microfungi from the open-savannah area were screened for antibiotic activity. Of these fungi, 28,1% (40 species) showed definite antimycotic activity against *Candida albicans*. A further 17 species showed a slight inhibition of growth of the latter fungus.

TABLE 2.

Taxonomic distribution of fungi with antifungal and antibacterial activity

Kinds of fungi	Number of species assayed	Antifungal activity	Percentage	Antibacterial activity*	Percentage
Phycomycetes	13	2	15,4	3	23,1
Ascomycetes	14	0	0	4	28,6
Aspergilli	15	6	40,0	8	53,3
Cladosporia	6	2	33,3	2	33,3
Fusaria	12	5	41,6	2	16,6
Penicillia	24	5	20,8	17	70,8
Trichodermae	11	8	72,7	10	90,9
Other Fungi Imperfecti	47	12	25,5	13	27,7
TOTALS	142	40	28,1	59	41,5

* Activity on agar media and in liquid media.

When assayed on agar media for antibacterial activity, 59 species of soil fungi (41.5%) showed positive antagonistic effects towards one or more of the bacteria. Only 9 of the isolates were found to be active against all the test bacteria. From Table 2 it is clear that species of *Trichoderma*, *Penicillium* and *Aspergillus* showed the highest percentage of species antagonistic against the bacterial test organisms.

The production of antibacterial substances by the soil fungi which showed positive results on agar cultures was confirmed in liquid-culture. Forty-nine of these fungal species were grown in liquid medium and culture filtrates were assayed for antibacterial activity. Twenty-eight species showed positive antagonism against *Bacillus subtilis*. It has been suggested that some of the positive antagonistic effects seen in agar culture techniques could be due to local changes in hydrogen ion concentration (Robbins, Kavanagh and Hervey, 1947) and not to true antagonism. Brian (1951) is, however, of the opinion that such false positive results are probably counter-balanced by the tendency of agar antagonism tests to give false negative results when the antibiotic produced is insoluble in water or of high molecular weight.

The only active species of Phycomycetes were *Absidia cylindrospora*, *Mucor fragilis* and *M. spinosus*. Species of these two genera were also the only active Phycomycetes from soils of Surrey and Dorset (Jeffreys *et al.*, 1953). In the Ascomycetes *Chaetomium globosum* var. *flavo-viride*, *C. lusitanicum*, *Gymnoascus umbrinus* and *Nectria invecta* showed definite antagonistic activity against test bacteria. Culture filtrates of *G. umbrinus* showed marked inhibition of *Bacillus subtilis*. Karow and Foster (1944) found species of this genus to be active producers of the antibiotic patulin. *Chaetomium* species are known to produce antibiotics (Waksman & Bugie, 1944).

Members of the genus *Aspergillus* have been extensively studied and most known species have been screened for antibiotic production, (Gill-Cary, 1949). Of the 15 *Aspergilli* assayed, only *Aspergillus niger*, *A. sydowii* and *A. wentii* were inactive. The most active species were *A. flaviceps*, *A. flavus*, *A. ochraceus*, *A. rugulosus* and *A. versicolor*, all of which are well known antibiotic producers (Raper & Fennell, 1965).

Species of *Gliocladium* are known to produce a variety of antibiotics (Brian, 1951). None of the species tested in this investigation were active. *Paecilomyces* species were also not active. *Penicillium chrysogenum*, *P. cyclopium*, *Penicillium sp.* (770), *P. sp.* (793), *P. sp.* (1041) and *P. sp.* (1168) were very active, showing antagonistic effects both on agar medium and in liquid culture. The outstanding fungus of this survey is certainly the ascosporous *Penicillium sp.* (770) which not only showed exceptional antibacterial activity, but also markedly inhibited growth of *Candida albicans*. Only four *Penicillium* species were inactive. The genus *Penicillium* is well known as a potential source of antibiotic substances

and the members of this genus have been assayed for antibiotics extensively (Brian, 1951; Raper & Thom, 1949).

It has been pointed out (Table 2) that species of *Trichoderma* were exceptionally antagonistic towards bacteria. The outstanding species here are *T. koningii*, *T. pseudokoningii*, *T. viride* and the unidentified species 1016, 1028 and 1066. The Dematiaceae family as a whole was not active. Species of the genera *Alternaria*, *Cladosporium*, *Curvularia* and *Stachybotrys* showed some antagonistic activity. *Stachybotrys atra* is known to produce antibiotics (Robbins, 1945). Of the rest of the Fungi Imperfecti, certain species of *Fusarium* and *Rhizoctona solani* showed some activity but *Fusarium solani* was the only species with marked antibacterial activity.

TABLE 3.

Percentage of abundant, common and rare species of soil fungi producing antibacterial and antifungal activity. (Abundant species—relative frequency more than 10%; common species = relative frequency 1–10%; rare species = relative frequency = less than 1%; Eicker, 1974.)

Fungus Species	Antibacterial activity	Antifungal activity
Abundant species		
<i>Mucor spinosus</i>	+	+
<i>Chaetomium globosum</i>	—	—
<i>Aspergillus rugulosus</i>	+	+
<i>Cladocidium roseum</i>	—	—
<i>Penicillium multicolor</i>	—	+
<i>Spicaria violaceae</i>	+	+
<i>Trichoderma viride</i>	+	+
<i>Fusarium</i> species (all species)	+	+
8 Species	63% active	75% active
Common species		
<i>Absidia cylindrospora</i>	—	—
<i>Cunninghamella echinulata</i>	—	+
<i>Mucor racemosus</i>	—	—
<i>Zygorhynchus moelleri</i>	—	—
<i>Chaetomium spinosum</i>	—	—
<i>Gymnoascus umbrinus</i>	+	—
<i>Pleospora infectoria</i>	—	—
<i>Coniella diplodiella</i>	+	—

Fungus species	Antibacterial activity	Antifungal activity
<i>Aspergillus carneus</i>	+	—
<i>A. flavus</i>	+	+
<i>A. fumigatus</i>	+	—
<i>A. versicolor</i>	+	+
<i>Paecilomyces</i> sp. (450)	+	—
<i>Paecilomyces</i> sp. (820)	+	—
<i>Penicillium chrysogenum</i>	+	—
<i>P. cyclopium</i>	+	—
<i>P. frequentans</i>	—	—
<i>P. rainstrickii</i>	—	—
<i>P. waksmanii</i>	+	—
<i>Scopulariopsis brevicaulis</i>	—	—
<i>Trichoderma pseudokoningii</i>	+	+
<i>Alternaria tenuissima</i>	+	—
<i>Cladosporium tenuissimum</i>	—	—
<i>Gonytrichum macrocladum</i>	—	—
<i>Humicola</i> sp. 309	—	—
<i>Stachybotrys atra</i>	+	—
<i>Epicoccum purpurascens</i>	—	—
<i>Doratomyces stemonites</i>	—	—
28 Species	44% active	14%
Rare fungi		
106 species	36% active	25% active

There appears to be a correlation between the capacity of a fungus to produce antibacterial and antifungal substances and its relative frequency of occurrence in the soil. Of the eight fungi most abundant in samples of the alkaline Transvaal soil 63% produced antibacterial substances and 75% produced antifungal substances (Table 3). In comparison, 44% of the common fungi (relative frequency 1–10%) produced antibacterial substances while only 14% of these fungi showed antifungal activity. The figures for the rare fungi are 36% and 25% for antibacterial and antifungal activity respectively. In spite of their abundance, certain fungi, such as *Chaetomium globosum* and *Gliocladium roseum* did not produce any antifungal or antibacterial antibiotics. *Penicillium multicolor*, also abundant, produced antifungal substances but showed no antibacterial activity. Previous investigations show that many of the widespread, non-producers of antibiotics are relatively resistant to antibiotics produced by other species (Jeffreys *et al* 1953; Dix, 1972) and this characteristic gives them a strong competitive ability.

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